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PERFORMING ORGANIZATION: **Yale University**
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14. ABSTRACT
The goal of this study was is to explore the biological activity of largazole, a cyclic depsipeptide natural product with remarkable potency in selectively inhibiting the proliferation of breast cancer cells without significant effects on normal breast mammary epithelial cells. Specifically, we set out to test the idea that dual-specificity of small molecules capable of targeting two or more aberrant signaling pathways associated with human cancers will be more efficacious in suppressing human tumors. This hypothesis was based on our initial observation that largazole and analogs inhibit both histone deacetylases and also the human ubiquitin E1 enzyme. Included in the scope of the research was the synthesis of additional quantities of largazole, studies to determine whether dual inhibition of both HDAC and ubiquitin conjugation is responsible for the selectivity of largazole against breast cancer cells and determine which HDAC isoforms render breast epithelial cells sensitive to largazole, and initial efforts to determine the chemotherapeutic efficacy of largazole to inhibit breast cancer growth and metastasis in mice.

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Nothing Listed

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TABLE OF CONTENTS

1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

The goal of this study was is to explore the biological activity of largazole, a cyclic depsipeptide natural product with remarkable potency in selectively inhibiting the proliferation of breast cancer cells without significant effects on normal breast mammary epithelial cells. Specifically, we set out to test the idea that dual-specificity of small molecules capable of targeting two or more aberrant signaling pathways associated with human cancers will be more efficacious in suppressing human tumors. This hypothesis was based on our initial observation that largazole and analogs inhibit both histone deacetylases and also the human ubiquitin E1 enzyme. Included in the scope of the research was the synthesis of additional quantities of largazole, studies to determine whether dual inhibition of both HDAC and ubiquitin conjugation is responsible for the selectivity of largazole against breast cancer cells and determine which HDAC isoforms render breast epithelial cells sensitive to largazole, and initial efforts to determine the chemotherapeutic efficacy of largazole to inhibit breast cancer growth and metastasis in mice.

2. **KEYWORDS:**

Breast cancer, ubiquitination, E1 activating enzyme, depsipeptide, natural product, histone deacetylase, HDAC, xenograft, 3D culture, SAR, triple-negative breast cancer

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

The proposal listed three major goals:

Aim 1. Synthesize derivatives of largazole with potentially improved molecular properties and improved selectivity for transformed *vs* non-transformed cells.

Aim 2. To determine whether dual inhibition of both HDAC and ubiquitin conjugation is responsible for the electivity of largazole against breast cancer cells and determine which HDAC isoforms render breast epithelial cells sensitive to largazole.

Aim 3. To determine the chemotherapeutic efficacy of largazole to inhibit breast cancer growth and metastasis in mice

Major Objectives and Activities

Three specific aims were proposed in the original application. Activities, progress, and results are described by each Aim below, and in the context of the approved SOW.

Aim 1. Synthesize derivatives of largazole with potentially improved molecular properties and improved selectivity for transformed *vs* non-transformed cells.

Task 1.1 Synthesize initial round of largazole analogs for SAR testing **(Phillips, Months 1-6) (Completed)**

Task 1.2 After initial SAR data is obtained, design and synthesize a second round of focused largazole analogs **(Phillips, Months 7-12) (Completed)**

Task 1.3 Write and submit manuscripts describing the intial phase of SAR studies as well as annual report to CDMRP **(Liu and Phillips, Month 12) (Completed)**

Task 1.4 Synthesize larger quantities of selected largazole derivatives for detailed testing **(Phillips, Months 14-16) (ongoing; completed for largazole)**

Task 1.5 Synthesize largazole derivatives targeted to specific cancer cells e.g. folic acid derivatives **(Phillips, Months 17-23) (ongoing)**

Task 1.6 Testing the activity of largazole derivatives using HDAC1 enzymatic assay, p27 ubiquitination and E1 thiolester assays **(Liu, Completed)**.

Results and Findings

Largazole stabilizes GFP-p27 expression in Kip16 cells. A hallmark of many advanced cancers is an excessive degradation of the cyclin-dependent kinase inhibitor p27, which is directed by SCF^{Skp2}-mediated ubiquitination. Hence, stabilization of p27 degradation represents a rational approach in cancer therapeutics. To identify small molecule inhibitors that can stabilize p27Kip1, we performed a screen of ~3000 compounds from NCI DTP diversity set along with several natural products in our collection. For the cell based screen, we generated a mink lung epithelial cell line (Kip16) stably expressing p27 that was cloned in frame with green fluorescent protein (GFP). The resulting N-terminal GFP-p27 fusion, detectable by fluorescence microscopy, was used to determine the levels of p27 expression upon treatment of cells with the compound libraries in 96-well format. Much to our surprise, the most potent hit that emerged from this screen was the natural compound largazole (Figure 1), which was first described by Luesch and coworkers (5) and subsequently synthesized in several laboratories including ours (1, 2, 3, 4, 5 6, 7). Largazole induced a robust and highly uniform upregulation of GFP-p27 at concentrations as low as 1 nM (Figure 2a) as compared to the expression levels after treatment with the proteasome inhibitor MG132.

We did not observe an increase in GFP-p27 expression upon treatment with the vehicle control DMSO. This result suggests that largazole can stabilize GFP-p27 expression in cultured cells.

Largazole and select analogues inhibit the *in vitro* ubiquitination of p27 and Trf1. Initial investigation into the mechanism of largazole indicated that the compound stabilized the expression of p27 in cells. Since the concentration of cyclin-dependent kinase inhibitor p27 is mainly regulated at the protein level by increased polyubiquitination and subsequent proteasomal degradation, we hypothesized that largazole and synthetic analogues stabilize p27 by inhibiting the ubiquitination pathway (8, 9). One of the downsides of cell based assays is that the effects observed may be attributed to the influence of multiple pathways. For example, inhibiting the proteasome, elevating transcription of GFP-p27, or inhibiting Cdk activity can also lead to an increase in p27 expression. To tease out the mechanism and action of largazole on p27 stabilization, we decided to test the effect of largazole on p27 ubiquitination in a fully reconstituted system *in vitro* (10, 11). To test if largazole affects p27 ubiquitination *in vitro*, we incubated largazole (L) with p27, ubiquitin E1, E2, SCF^{Skp2}, and Cks1. As shown in Figure 1, adding largazole significantly reduced polyubiquitinated p27, suggesting that largazole can block p27 ubiquitination. Since largazole is known to be a histone deacetylase inhibitor and has a thioester moiety that links an aliphatic chain to the core, we decided to test whether inhibition of p27 degradation can be linked to its histone deacetylase inhibitory activity. The structure-activity relationship for largazole is relatively well understood (12). Therefore we next tested a series of largazole analogues to get a preliminary structure-activity relationship on p27 ubiquitination. To investigate this, largazole ester (E), largazole ketone (K), largazole macrocycle (M), and *seco*-largazole (S) were tested in an *in vitro* p27 ubiquitination assay (Figure 2b). We also added the HDAC inhibitor Trichostatin A (TSA), the structure of which can be found in Figure 1, to the assay to determine whether or not other HDAC inhibitors affect p27 ubiquitination. We observed that largazole (L), largazole ketone (K), and largazole ester (E) inhibited the ligation of ubiquitin onto p27; however, the M and S analogues and TSA failed to inhibit the ubiquitination of p27 (Figure 1). This result suggests both the macrocycle and aliphatic chain are required for ubiquitin E1 inhibition. Furthermore, the result also suggests that the thioester moiety of largazole largazole is not required for inhibition, because the ketone and ester analogues were equally potent in blocking p27 ubiquitination. In addition, E1 inhibition is unrelated to HDAC inhibitor activity of largazole as both ketone and ester failed to inhibit HDAC and TSA, a known HDAC inhibitor, does not block p27 ubiquitination *in vitro*. Prior to ubiquitination, p27 is phosphorylated by the Cdk2-CyclinE complex.

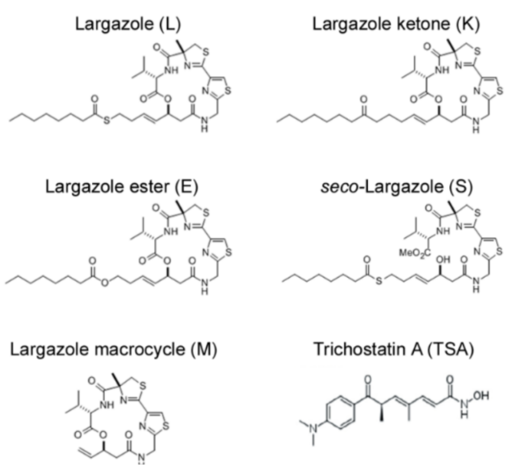

Figure 1. Chemical structures of Largazole, synthetic analogues synthesized and Trichostatin A.

We carried out an *in vitro* p27 phosphorylation assay in the presence of either DMSO or largazole in order to test whether or not the decrease in p27 ubiquitination was due to the inhibition of the

Cdk2-CyclinE complex. We observed that largazole does not inhibit the phosphorylation of p27 (Figure 2b); therefore, the inhibition of p27-ubiquitin conjugation is due to an inhibition of the ubiquitination process rather than phosphorylation step.

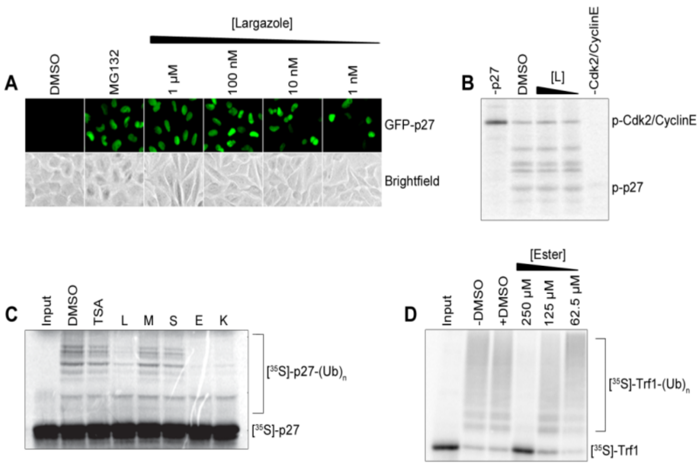

Figure 2. Largazole stabilizes p27 expression in Kip16 cells and inhibits p27 ubiquitination *in vitro* but not phosphorylation of Cdk2-CyclinE*.*

Even though there are a limited number of proteins in the reconstituted p27 ubiquitination system *in vitro*, tracing the real target of largazole is challenging. Fortunately, we have previously established another reconstituted *in vitro* ubiquitination assay of Trf1 with SCF^{Fbx4} (13). There are a few overlapping components between these two assays. The effect of largazole on Trf1 ubiquitination should offer some insight as to where largazole might target. To study the specificity of Largazole, we added largazole ester to an *in vitro* Trf1 assay and found that largazole ester inhibited the ligation of ubiquitin onto Trf1 in a dose-dependent fashion. Since Trf1 and p27 require different E2 ubiquitin-conjugating enzymes and different E3 ubiquitin-ligating recognition subunits in order to carry out each ubiquitination, we hypothesized that largazole and select synthetic analogues inhibit a step common to both ubiquitination pathways.

Largazole ketone inhibits ubiquitin E1 activation. In vertebrates, there exists only one known ubiquitin-activating E1 enzyme, UBA1. Since both p27 and Trf1 can be ubiquitinated in the presence of UBA1, we hypothesized that the inhibitory activity of largazole is due to the deactivation of E1. To test this hypothesis, we incubated largazole and largazole ketone with recombinant E1 prior to carrying out an *in vitro* thioester assay we described previously (14). The presence of a fluorescence signal in the thioester assay suggests the formation of E1-ubiquitin adducts. The dose dependent decrease in fluorescence indicates that largazole and largazole ester inhibit the formation of E1-ubiquitin adducts (Fig 3AC). The dose-response curves generated from Figure 3BD suggest an IC_{50} of approximately 29 µM and 25 µM, respectively.

Activated ubiquitin is normally transferred to ubiquitin conjugating enzymes (E2). If E1 activity is inhibited, we expect to see the attachment of ubiquitin onto Cdc34 (E2) to be impaired. To

further validate E1 inhibition, we included Cdc34, the E2 enzyme required for p27 ubiquitinaton, in the E1 reaction mixture. As shown in Figure 3EF, in the presence of ATP, fluorescent ubiquitin is transferred to Cdc34 indicated by the presence of a fluorescent Cdc34 band on the gel. Upon incubation with E2, largazole or largazole ester reduce the amount of ubiquitin molecules that are transferred from E1 to E2 in a dose-dependent fashion. This result is consistent with the notion that largazole or largazole ester inhibit E1 activity.

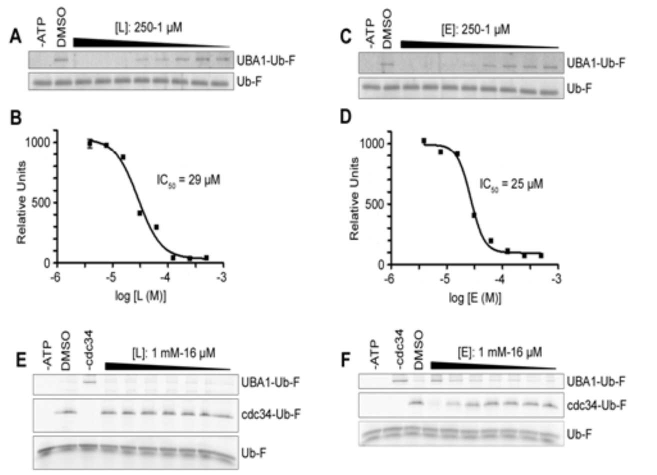

Figure 3. Largazole (L) and largazole ester (E) inhibit ubiquitin E1 in a dose dependent manner in vitro.

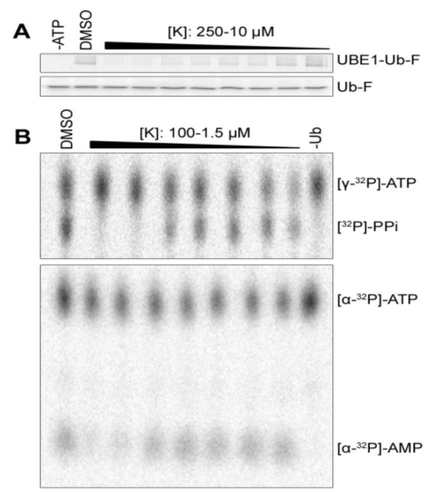

Figure 4. Largazole ketone inhibits the adenylation of the E1 ubiquitin-activating enzyme.

A potential caveat of the above experiment is that if largazole or largazole ester also blocks the transfer of ubiquitin from E1 to E2 we would have seen the same result. To rule out this possibility, we first produced ubiquitin charged E1 by incubating ATP and fluorescent ubiquitin for 30 min at room temperature followed by the addition of Cdc34, which was also contained with largazole or largazole ester. If either compound blocks ubiquitin transfer from E1 to E2, we would observe a significant decrease in Cdc34 fluorescence signal regardless of the order of compound addition. On the other hand, we should see the opposite results. As shown in Figure 3EF, Cdc34 is fully conjugated with fluorescence ubiquitin when largazole or largazole ester was added after generating fluorescent ubiquitin-E1. This result suggests that largazole or largazole ester neither blocks the transfer of activated ubiquitin from E1 to E2 nor promotes hydrolysis of ubiquitin thioester.

Largazole ketone inhibits the adenylation step of E1 activation. E1 forms an ubiquitin–adenylate intermediate during the course of its catalytic cycle (15). Thus the mechanism of ubiquitin E1 activation can be studied by assaying ATP:PPi and AMP:ATP exchanges (15). Production of AMP in the [α-^{32}P]-AMP:[α-^{32}P]-ATP exchange assay guarantees that a thioester bond is formed between E1 and ubiquitin, while the release of PPi, measured by the [^{32}P]-PPi:[γ-^{32}P]-ATP exchange assay, signals the formation of ubiquitin adenylate. To further dissect the mechanism of largazole inhibition, two nucleotide exchange assays were carried out in the presence of largazole derivatives. For these experiments we used largazole ketone, which is similar to largazole and largazole ester. From the results shown in Figure 4, it is evident that the

first two concentrations of largazole ketone (100 and 50 μM) inhibit ubiquitination of E1 similarly and were also inhibitory in both types of exchange assays. The lack of a [^{32}P]-PPi signal suggests that the adenylation step did not occur; consequently, ubiquitin could not be transferred to the active site cysteine to trigger the release of AMP. Both steps of the E1-catalyzed reactions can be measured by the AMP:ATP exchange assay. The lack of an [α-^{32}P]-AMP signal further suggests that the adenylation step is inhibited by largazole ketone. Thus largazole or largazole derivatives act on the first step of ubiquitin activation pathway by blocking the formation of ubiquitin-adenylate.

Selectivity of largazole ketone against SUMO E1 and Uba1p. In addition to ubiquitin, there exist several ubiquitin-like proteins that covalently modify other proteins. All of the ubiquitin-like proteins have activation pathways similar to ubiquitin (16). In order to study the specificity of largazole to the ubiquitin pathway, we incubated largazole ketone with SUMO-activating E1 enzyme prior to carrying out a thioester assay. From the results in Figure 5B, we found that largazole ketone is ineffective in inhibiting the formation of E1-SUMO adducts. From the dose-response curve generated from the SUMO E1 fluorescence results, the IC_{50} is approximately 450 μM as opposed to 10 μM for ubiquitin E. Thus largazole is relatively selective in perturbing ubiquitin E1 activation.

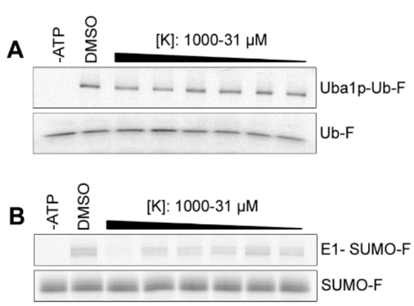

Figure 5. Investigation into the selectivity of Largazole ketone (K). A, Largazole ketone (K) fails to inhibit the ligation of ubiquitin onto Uba1p, a homologue of UBA1 from *S. pombe*. Formation of Uba1p-ubiquitin adducts was determined by thioester assay utilizing fluorescein-ubiquitin.

Ubiquitin and the ubiquitin E1 enzyme are highly conserved among eukaryotes (16). Sequence analysis shows a 45% homology between the human ubiquitin-activating enzyme E1 (UBA1) and S. pombe E1 (ptr3/Uba1p) at the amino acid sequence level. To test whether largazole ketone inhibits the S. pombe E1, we carried out a thioester assay using largazole ketone and the ubiquitin E1 homologue in *S. pombe*, Uba1p. The results in Figure 5A suggest that largazole ketone fails to inhibit the formation of E1-ubiquitin adducts at concentrations less than 1 mM. Taken together, these results suggest that largazole and its derivative are highly selective in inhibiting the ubiquitin E1 enzyme.

The results obtained in Aim 1 have been published (see **6. Products** below, and dissemination section).

Aim 2. To determine whether dual inhibition of both HDAC and ubiquitin conjugation is responsible for the selectivity of largazole against breast cancer cells and determine which HDAC isoforms render breast epithelial cells sensitive to largazole.

We have started the effort of cloning of all HDAC enzymes. So far we have cloned HDAC1, 2, 3, 4, 6, 8 and 11. We will continue to procure clones from ATCC to complete the HDAC enzyme set cloning project. The TGI assay on MDA-MB231 and a battery of breast cancer cell lines have been ongoing.

Task 2.1 Cloning of 17 HDAC enzymes into lentiviral vector **(Liu, Months 1-4) (Completed).**

Task 2.2 Construct human mammary epithelial cell lines (HME) expressing each individual HDAC enzyme **(Liu, Months 5-16) (Partially completed).**

Task 2.3 Perform cell based growth inhibition assays using MDA-MB231 and HME cells using largazole analogs generated in Aim 1 **(Liu, Months 6-18) (ongoing).**

Task 2.4 Measure cell permeability of lagarzole analogs using parallel artificial membrane permeability assay **(Liu, Months 6-18) (completed).**

Task 2.5 Measuring the largazole sensitivity of HME cell lines expressing HDAC enzymes **(Liu, Months 12-18) (Completed).**

Task 2.6 Perform siRNA and shRNA experiments for informative HDAC enzymes in HME and MDA-MB231 cells **(Liu, Months 19-24) (completed).**

Task 2.7 Write the manuscript describing dual targeting activity of largazole **(Liu and Phillips, Month 12, completed).**

We tested a panel of 18 breast cancer cell lines from a heterogeneous group of breast cancer cell lines. In our initial exploratory studies, we made an interesting observation that triple negative breast cancer (TNBC) cell lines are particularly sensitive to Paragazole inhibition. Since TNBC are associated with a shorter median

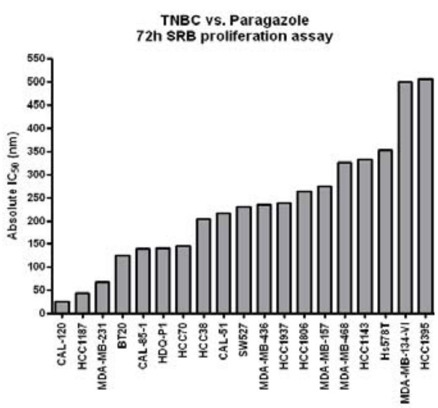

Figure 6.Growth Inhibition assays of Paragazole in TNBC lines.

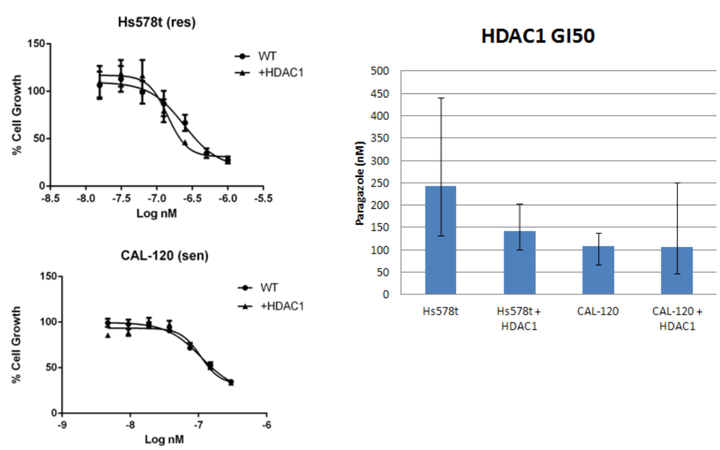

Figure 7. Growth Inhibition assays of Largazole sensitivity in cell lines overexpression of HDAC1.

time to relapse and death and significant unmet medical need due to the fact that these cancers do not respond to endocrine therapy or other available targeted agents, we decide to focus on testing TNBC cell lines instead of a broader spectrum of breast cancer cell lines. We used a panel of 19 breast cancer cell lines to assess the proliferative response to increasing concentrations of Paragazole using an SRB assay. As shown in Figure 6, a majority of these cell lines are quite sensitive (<IC50=500 nM) to Paragazole treatment although some are more sensitive than others. Several TNBC lines are inhibited at subnanomolar concentrations of

Paragazole. These preliminary data suggests that Paragazole might be a novel agent to combat TNBC.

Next we picked a sensitive cell line CAL-120 and a relatively growth resistance cell line Hs578t to stably over express HDAC1,3,6,8 in them using lentiviral mediate gene transfer. Of four enzymes, only overexpression of HDAC1 has observable differences in largazole or Paragazole response. These differences are quite small and more rigorous studies are needed to establish that the effects are reproducible and statistically significant (Figure 7). Preliminary data does appear to support the hypothesis that overexpression of certain HDAC isoform may render cells more sensitivity to largazole inhibition.

Aim 3. To determine the chemotherapeutic efficacy of largazole to inhibit breast cancer growth and metastasis in mice

Task 3.1 Large scale synthesis for largazole for initial testing along with control (Taxol) **(Phillips, Months Completed)**
Task 3.2 Large scale synthesis of the improved lead for animal testing **(Phillips, Completed)**
Task 3.3 Determining the chemotherapeutic effectiveness of largazole to prevent mammary tumor growth, invasion, and angiogenesis using the xenograft animal model **(Schiemann, Completed)**
Task 3.4 Assessing the effects of largazole to prevent mammary tumor metastasis **(Schiemann, Completed)**
Task 3.5 Testing newly improved largazole analogs in growth, invasion, agiogenesis and tumor metastasis in the xenograft animal model **(Schiemann, Months 12-23)**
Task 3.6 Write and submit manuscripts for publication and final report to CDMRP **(Liu, Phillips and Schiemann, Month 24)**

Because Dr. William Schiemann, the collaborator on this project has moved his laboratory to Case Western Reserve University in Cleveland, it took a while for the BRCP to complete issuing the funding for this aim to us. Because of his move we have to rework the subcontract and obtain approval for IACUC and animal protocols. In addition, Professor Schiemann had to rebuild his research team at Case Western Reserve University since many experienced researchers in his lab did not follow him to Cleveland. Despite of these setbacks, Professor Schiemann group managed to make progress on this project. We made a derivative of largazole called Paragazole. In the last report, we mentioned that Professor Schiemann had obtained preliminary results showing that 1) Paragazole Potently Inhibits the Growth of Human and Murine TNBC Organoids (Figure 8); 2) Paragazole Potently Inhibits the Growth of Human and Murine TNBC Organoids (Figure 8). We requested a no cost extension to allow us to complete the remaining SOW. This includes repeat in vivo tumor suppression experiments. During the no cost extension period, we have shown that Paragazole (10 mg/kg) was highly effective at inhibiting the development and metastasis of human MDA-MB-231 tumors in nude mice. Importantly, the lymph node metastasis of MDA-MB-231 cells was completely absent in Paragazole treated animals (Figure 9).

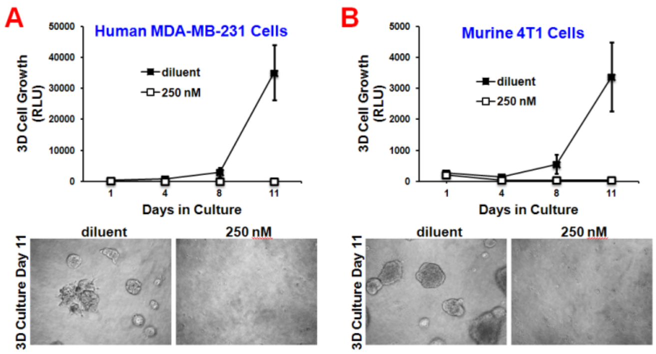

Fig. 8. Paragazole potently inhibits the growth of human and murine TNBC organoids. Human MDA-MB-231 (A) a murine 4T1 (B) TNBC cells were incubated in the absence (diluent) or presence of increasing concentrations Paragazole (0-2 mM) over a span of 11 days. Differences in organoid growth were measured longitudinally bioluminescence *(top),* or by bright-field microscopy *(bottom).* Data are the mean (+/-) STD of organoids trea with 250 nM of Paragazole.

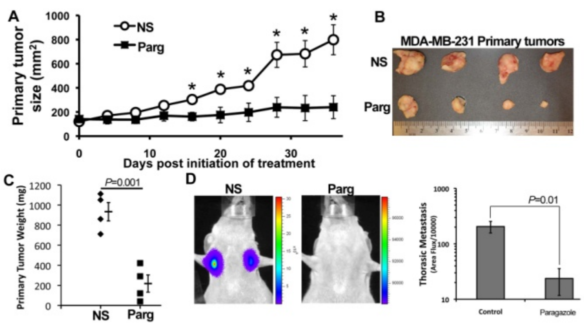

Fig. 9. Paragazole inhibits human TNBC tumor growth and metastasis in nude mice. Human MDA-MB-231 cells million cells/injection) were engrafted onto the mammary fat pads of nu/nu mice and allowed to develop until tumors reached a size of 400 mm3, at which point cohorts were treated with diluent (NS), Paragazole (10 mg/ Paragazole). (A) tumor growth curve (B) size of tumors (C) Weight of tumor (D) imaging of growing tumor in anim (E) metastasis.

Overall, our studies clearly demonstrated these largazole and aragazole are very promising in treated triple negative breast cancers in vitro and in vivo.
A manuscript describing this work is currently in preparation.

What opportunities for training and professional development has the project provided?

Nothing to Report.

How were the results disseminated to communities of interest?

The results of the research conducted as part of this award was disseminated via one joint publication:

1. Ungermannova D, Parker SJ, Nasveschuk CG, Wang W, Quade B, Zhang G, Kuchta RD, Phillips AJ, Liu X. Largazole and its derivatives selectively inhibit ubiquitin activating enzyme (e1). PLoS One. 2012;7(1):e29208.

and two additional publications that described research that was related:

2. Cole KE, Dowling DP, Boone MA, Phillips AJ, Christianson DW. Structural basis of the antiproliferative activity of largazole, a depsipeptide inhibitor of the histone deacetylases. J Am Chem Soc. 2011 Aug 17;133(32):12474.
3. Chapnick DA, Jacobsen J, and Liu, X. The development of a novel high throughput computational tool for studying individual and collective cellular migration. PLoS One. PLoS One. 2013: 8(12):e82444.

In addition, PI Phillips has described the results of this research in >10 talks at academic institutions.

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to Report.

4. IMPACT:

In this research we used a combination of synthetic organic chemistry, cell-biology and biochemical mechanistic studies to study the effect of the natural product largazole and related compounds on breast cancers. Our research demonstrated three key points:

a. That Largazole and its analogs selectively inhibit ubiquitin E1 enzyme activity in vitro. This enzyme is responsible for the activation of ubiquitin – one of the cell's tags for proteasomal degradation – in the first step of a three-step process.
b. We show that Largazole inhibition of E1 is highly selective as it does not inhibit a highly related ubiquitin E1 enzyme from the yeast S. pombe and is almost twenty fold less effective in inhibiting the activation of SUMO E1.
c. We show that Largazole and Paragazole potently inhibit the growth of human and murine triple negative breast cancer organoids and rhat *Largazole and Paragazole exhibit cytotoxicity against Human MDA-MB-231 tumors in mice.*

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

The University of Colorado, along with co-PI Xuedong Liu, formed a company based in part on largazole dervivatives: OnKure Inc (Longmont, CO).

What was the impact on society beyond science and technology?

Nothing to Report

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report.

5. CHANGES/PROBLEMS: The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

In the last report, we mentioned that Professor Schiemann had obtained preliminary results showing that Paragazole Potently Inhibits the Growth of Human and Murine TNBC Organoids. We requested a no cost extension to allow us to complete the remaining SOW. This includes repeat in vivo tumor suppression experiments. During the no cost extension period, we have shown that Paragazole (10 mg/kg) was highly effective at inhibiting the development and metastasis of human MDA-MB-231 tumors in nude mice. Importantly, the lymph node metastasis of MDA-MB-231 cells was completely absent in Paragazole treated animals .

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. PRODUCTS:

Publications, conference papers, and presentations
Journal publications.

The results of the research conducted as part of this award was disseminated via one joint publication:

1. Ungermannova D, Parker SJ, Nasveschuk CG, Wang W, Quade B, Zhang G, Kuchta RD, Phillips AJ, Liu X. Largazole and its derivatives selectively inhibit ubiquitin activating enzyme (e1). PLoS One. 2012;7(1):e29208. **Support acknowledged.**

and two additional publications that described research that was related:

2. Cole KE, Dowling DP, Boone MA, Phillips AJ, Christianson DW. Structural basis of the antiproliferative activity of largazole, a depsipeptide inhibitor of the histone deacetylases. J Am Chem Soc. 2011 Aug 17;133(32):12474.
3. Chapnick DA, Jacobsen J, and Liu, X. The development of a novel high throughput computational tool for studying individual and collective cellular migration. PLoS One. PLoS One. 2013: 8(12):e82444.

Books or other non-periodical, one-time publications.

Nothing to Report.

Other publications, conference papers, and presentations.

Nothing to Report.

Website(s) or other Internet site(s)

Nothing to Report.

- **Technologies or techniques**
  Nothing to Report.

- **Inventions, patent applications, and/or licenses**

  Nothing to Report.

- **Other Products**

  Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name: Andrew J. Phillips
Project Role: PI
Researcher Identifier (e.g. ORCID ID): AJPHILLIPS (NIH COMMONS)
Nearest person month worked: 3
Contribution to Project:Dr. Phillips provided leadership and guidance to project direction.
Funding Support: W81XWH-10-1-0990

Name:Suk Youn Kang
Project Role: Postdoctoral Associate
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 8.5
Contribution to Project: Dr. Kang was part of the team that provided larger amounts of largazole and synthetic analogs.
Funding Support: W81XWH-10-1-0990

Name:Jason Rush
Project Role: Postdoctoral Associate
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 2.5
Contribution to Project: Dr. Rush contributed to the analysis of data on the activity of largazole and largazole aalogs.
Funding Support: W81XWH-10-1-0990

Name:Michael Hallside
Project Role: Postdoctoral Associate
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 2.25
Contribution to Project: Dr. Hallside was part of the team that provided larger amounts of largazole and synthetic analogs
Funding Support: W81XWH-10-1-0990

Name:Shannon Carpenter
Project Role: Graduate Research Assistant
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 1.5
Contribution to Project: Ms. Carpenter contributed to the synthesis of larger amounts of largazole and synthetic analogs.
Funding Support: W81XWH-10-1-0990

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

Organization Name: University of Colorado
Location of Organization: Boulder, CO 80309
Partner's contribution to the project: biological assays and mechanism of action questions.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

This is a report that is largely duplicative of co-PI Xuedong Liu's report, notwithstanding formatting differences. The research performance is clearly marked in the Aims of the incorporated SOW.

QUAD CHARTS: N/A

9. APPENDICES:

Appendix 1: References

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Appendix 2. Publication (Ungermannova et al. PLoS One as detailed in **6. Products**)

Largazole and Its Derivatives Selectively Inhibit Ubiquitin Activating Enzyme (E1)

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Abstract

Protein ubiquitination plays an important role in the regulation of almost every aspect of eukaryotic cellular function; therefore, its destabilization is often observed in most human diseases and cancers. Consequently, developing inhibitors of the ubiquitination system for the treatment of cancer has been a recent area of interest. Currently, only a few classes of compounds have been discovered to inhibit the ubiquitin-activating enzyme (E1) and only one class is relatively selective in E1 inhibition in cells. We now report that Largazole and its ester and ketone analogs selectively inhibit ubiquitin conjugation to $p27^{Kip1}$ and TRF1 *in vitro*. The inhibitory activity of these small molecules on ubiquitin conjugation has been traced to their inhibition of the ubiquitin E1 enzyme. To further dissect the mechanism of E1 inhibition, we analyzed the effects of these inhibitors on each of the two steps of E1 activation. We show that Largazole and its derivatives specifically inhibit the adenylation step of the E1 reaction while having no effect on thioester bond formation between ubiquitin and E1. E1 inhibition appears to be specific to human E1 as Largazole ketone fails to inhibit the activation of Uba1p, a homolog of E1 in *Schizosaccharomyces pombe*. Moreover, Largazole analogs do not significantly inhibit SUMO E1. Thus, Largazole and select analogs are a novel class of ubiquitin E1 inhibitors and valuable tools for studying ubiquitination *in vitro*. This class of compounds could be further developed and potentially be a useful tool in cells.

Citation: Ungermannova D, Parker SJ, Nasveschuk CG, Wang W, Quade B, et al. (2012) Largazole and Its Derivatives Selectively Inhibit Ubiquitin Activating Enzyme (E1). PLoS ONE 7(1): e29208. doi:10.1371/journal.pone.0029208

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Introduction

In humans, protein ubiquitination is a dynamic process, depending on a tightly regulated balance between the activity of two ubiquitin-activating enzymes (E1s), approximately 40 ubiquitin-conjugating enzymes (E2s), and hundreds of ubiquitin ligases (E3s). Protein ubiquitination and subsequent degradation regulates almost every aspect of eukaryotic cellular function including cell cycle regulation, endocytosis, signal transduction, apoptosis, DNA damage repair, transcriptional regulation, and many others [1]. Hershko and coworkers discovered that ubiquitin covalently modified proteins prior to their degradation in rabbit reticulocyte lysates and characterized the reaction mechanism [2]. They first described the ubiquitin-activating enzyme, E1, that carries out the ATP-dependent activation of the C-terminal glycine residue of ubiquitin prior to ligation. In the first step of the E1 activation, the enzyme forms a complex with ubiquitin and ATP and catalyzes the adenylation of ubiquitin and successive release of pyrophosphate (PPi). During the second step, a thioester bond is formed between the C-terminus of ubiquitin and E1, subsequently releasing adenosine monophosphate (AMP). In the final step of E1 activation, additional ATP and ubiquitin are recruited to the adenylation site, generating a fully loaded E1 carrying two molecules of ubiquitin. The activated ubiquitin is then transferred to a cysteine in the active site of ubiquitin carrier protein E2, also via thiol ester linkage. Some E2 enzymes transfer ubiquitin to acceptor proteins directly, whereas other E2s require additional substrate binding proteins known as ubiquitin ligases or E3s [3,4].Through this mechanism, ubiquitin is attached to proteins by isopeptide linkages between the C-terminal Gly76 of ubiquitin and the ε-amino groups of lysine residues present in substrate proteins. In addition, linkages between Lys48 of one ubiquitin and the C-terminal Gly76 of another ubiquitin ultimately form polyubiquitin chains [5]. Once polyubiquitinated, proteins are targeted by the 26S proteasome for degradation.

In many human cancers, the ubiquitination system is often destabilized. For example, the cyclin-dependent kinase inhibitor p27 is mainly regulated at the protein level and is excessively degraded in approximately 50% of all human cancers [6,7]. Furthermore, expression of p27 is primarily controlled by polyubiquitination via the SCF^{Skp2} E3 ubiquitin ligase and subsequent proteasomal degradation [8]. The SCF^{Skp2} is a cullin-RING ligase (CRL), which is comprised of RING-box protein I (Rbx1), scaffold protein Cul1, linker protein Skp1, and F-box protein Skp2 [9]. In order for the ligase to function, Cul1 must first be covalently modified by NEDD8, an ubiquitin-like protein

[10–12]. Therefore, an observed stabilization of p27 in cells could result from decreased polyubiquitination by inhibiting the neddylation of Cul1 or one of the enzymes required for ubiquitination.

Given that ubiquitination influences many cellular functions, malfunctions in the pathway play a role in the pathogenesis of human neurodegenerative disorders such as Parkinson's, Alzheimer's and Huntington's diseases, as well as cancer. Inhibiting components of the ubiquitination system seems to be an avenue of therapeutic development with clinical applications [13,14]. For example, each E3 ligase targets a small number of proteins for ubiquitination, which makes it a potential target for highly specific inhibitors that have few side effects. There has, however, been little success in developing inhibitors of specific E3 ligases until recently [14,15]. Also, proteasome-inhibiting compounds have been a target of interest and were originally developed as tools for probing its proteolytic function [16,17]; however, these inhibitors were considered as possible cancer therapeutics after it was observed that they induced apoptosis in leukemic cell lines [18–20]. Although inhibiting the proteasome would nonspecifically inhibit the entire ubiquitination system, the proteasome inhibitor Bortezomib has fared surprisingly well in clinical trials and is now FDA approved for the treatment of relapsed and refractory myeloma and mantle cell lymphoma [19]. Therefore, inhibitory compounds of the ubiquitin system, whether they are specific or nonspecific, have the potential to be important therapeutics for the treatment of cancer.

In January 2008, the Leusch group at the University of Florida identified a natural product they named Largazole, which was isolated from cyanobacteria of the *Symploca* genus. They examined the compound for cytoxicity against cancer cells and observed remarkable antiproliferative activity in transformed mammary epithelial cells. In addition, they showed that Largazole preferentially targets cancer cells over normal cells, which makes this marine substance an important synthetic target as well as a potentially valuable cancer chemotherapeutic. Remarkably, the structure consists of several unusual features, such as a 16-membered macrocycle containing a 4-methylthiazoline fused to a thiazole ring and an octanoic thioester side chain, a unit rarely found in natural products. [21]. Also, Leusch and co-workers first reported the total synthesis of Largazole and determined that the molecular basis for its anticancer activity is HDAC inhibition [21,24]. Numerous analogs of Largazole have been generated in efforts to understand the structure-activity relationship, and it has been determined that the thioester moiety is required for HDAC inhibition [21–32]. Here, we report *in vitro* mechanistic studies that reveal a potential role of Largazole as an antagonist of the ubiquitin-activating enzyme E1. In contrast to HDAC inhibition, ketone and ester analogs of Largazole can actively block the ligation of ubiquitin onto E1, indicating a differential mode of inhibitory activity since the formation of a thiol metabolite is indispensible for E1 inhibition. More explicitly, Largazole's presence negatively affected the formation of ubiquitin adenylate, which we monitored through nucleotide exchange assay.

Materials and Methods

Construction of Kip16, a GFP-p27 Expressing Cell Line

Mink lung epithelial cells expressing GFP-p27 were generated by retroviral-mediated gene transfer. pBabe-GFP-p27 amphotropic virus was made by cotransfecting pBabe-GFP-p27-Puro with pCL-Ampho in 293T cells. Viral supernatant was collected and used to infect mink lung epithelial cell line Mv 1 Lu (CCL-64) from ATCC in the presence of 8 μg/ml polybrene. Puromycin was added at 5 μg/ml and stable clones were selected. Each clone was subcultured and tested for GFP-p27 expression in the presence or absence of 10 μM MG132 (Calbiochem, Darmstadt, Germany) for 24 hours. Clones expressing high levels of GFP in the presence of MG132 but low or unndetectable GFP in its absence were expanded. Immunoblotting using an anti-p27 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used to confirm the expression of the GFP-p27 fusion protein and stabilization of GFP-p27 upon MG132 treatment. One of the clones used for all subsequent studies was named Kip16.

Largazole Treatment of Kip 16 cells

Total synthesis of Largazole and Largazole analogs is described in [24] within the supporting information (including copies of spectra of all compounds) and is available at http://pubs.acs.org. Kip16 cells were seeded into 96-well flat clear-bottomed plates at 40,000 cells/well in 100 μl medium and incubated overnight at 37°C in a humidified 5% CO2 atmosphere. Largazole was then added to final concentrations ranging from 1 μM to 1 nM in 300 μl of fresh medium. 0.3% DMSO and 1 μM of MG132 were used as negative and positive controls, respectively. After 24 hours of incubation, the medium was removed, the cells were washed twice with phosphate-buffered saline (PBS), and the cells were fixed with 4% paraformaldehyde in PBS for 15 minutes and stored at 4°C for microscopy evaluation. Cells were visualized with a GFP filter set using a 10× objective on an Eclipse TE2000-S (Nikon, Melville, NY) equipped with a Photometrics camera (Roper Scientific, Tucson, AZ).

UBA1 and His-cdc34 Purification

Human ubiquitin E1 (UBA1) was expressed with an N-terminal GST tag fusion by means of recombinant baculovirus expression in Hi5 insect cells using the pFastBacHTA vector (Invitrogen, Carlsbad, CA). The cells were lysed by sonication in the presence of protease inhibitors in a buffer containing 200 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% NP40, 1 mM DTT, and 1 mM EDTA. Cleared lysate was incubated with glutathione beads (Amersham, Sweden) for one hour at 4°C. After three washes with lysis buffer, untagged E1 was produced by thrombin cleavage. The protein solution was passed through a S200 gel filtration column (Amersham, Sweden), and UBA1 concentration and purity was evaluated by SDS-PAGE and Coomassie Blue gel staining. The purity was generally greater than 90% and purified UBA1 was aliquoted and stored at −80°C after quick freezing in liquid nitrogen. N-terminal hexahistidine (His)-tagged human Cdc34 was cloned into the pQE-30 vector (Qiagen, Valencia, CA) and expressed in *Eschericia coli*. His-cdc34 was purified by Ni-NTA chromatography followed by ion exchange and size-exclusion chromatography. The purity and concentration of His-cdc34 were determined by SDS-PAGE analysis.

In Vitro Ubiquitination of p27 and Trf1

Mouse p27, cloned into pCS2, was translated *in vitro* in a reticulocyte lysate system (Promega, Madison, WI) in the presence of [^{35}S]-labeled methionine. p27 was phosphorylated by purified recombinant Cdk2-CyclinE as outlined by Ungermannova et al [33]. 5 μl of the phosphorylation reaction was incubated with a ubiquitin mixture containing 100 nM UBA1, 200 nM His-cdc34, 100 nM SCF^{Skp2} E3 ligase complex, 50 nM Cks1, 10 μM ubiquitin (Sigma Aldrich, St. Louis, MO), 10 μM methylated ubiquitin (Boston Biochem, Cambridge, MA), 1 μl of energy regeneration system (noted as 20×ER and consisting of 10 mM ATP, 20 mM Tris-HCl pH 7.4, 100 mM $MgCl_2$, 200 mM creatine phosphate, 2 mg/ml creatine phosphokinase, and 10%

glycerol) , 1 μM ubiquitin aldehyde, and 1 μM MG132 in a total volume of 15 μl. The reaction was quenched after 30 minutes in a 30°C water bath by addition of 4× SDS sample buffer. The products of ubiquitination were resolved by SDS-PAGE, destained in a 45% methanol and 10% acetic acid solution in water, dried and exposed overnight to a phosphoimager screen, and scanned using a Typhoon scanner 9400 (GE Healthcare, Piscataway, NJ). *In vitro* Trf1 ubiquitination was carried out as described in Zeng et al [34]. Recombinant Trf1, labeled with [γ-^{33}P]-ATP by CDK1-CyclinB, was incubated with 0.5 μM UBA1, 5 μM UbcH5a, 1 μM SCF^{Fbx4} E3 ligase complex, 5 μM ubiquitin, 100 μM methylated ubiquitin, 1 μM ubiquitin aldehyde, and 1 μl 20×ER for two hours at 30°C. Ubiquitinated Trf1 was analyzed by SDS-PAGE followed by autoradiography.

E1/E2 Thioester Bond Formation Assay

40 nM ubiquitin E1 (UBA1) or 1 μM *S. pombe* E1 (Uba1p, gift from Chris Lima) or 0.5 μM human SUMO E1 (Boston Biochem, Cambridge, MA) were pre-incubated with 20×ER at 30°C for 5 minutes in thioester reaction buffer (20 mM Tris pH 7.6, 50 mM NaCl, and 10 mM $MgCl_2$). After 5 minutes, 1 μM fluorescein-ubiquitin (Boston Biochem, U-590) was added to initiate attachment of ubiquitin. All components were allowed to react for another 5 minutes in a total volume of 5 μl. The reaction was stopped with 10 μl of SDS-PAGE loading buffer, minus DTT, and the proteins were resolved using 12% gels that were run on ice to prevent the reduction of E1-ubiquitin due to the heat generated during electrophoresis. Thioester bond formation was visualized by scanning the gel using Typhoon scanner 9400 (GE Healthcare) that was set to fluorescence mode (532 nM). When necessary 100 nM of E2 (Cdc34) was added after the E1 enzyme was pre-charged with ATP. Serially diluted Largazole and its analogs were incubated with the reagents as stated in the text. ImageJ was utilized to quantify the fluorescence signal, and the dose response curves were generated by nonlinear least regression analysis of data using Prism (GraphPad, San Diego, CA).

[α-^{32}P]-AMP: [α-^{32}P]-ATP and [^{32}P]-PPi:[γ-^{32}P]-ATP Exchange Assays

The reaction mixture contained, in a final volume of 10 μl, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$ (reaction assay buffer), 150 nM human ubiquitin E1 (UBA1), 100 μM ATP, 2 mM AMP, 1 μM [α-^{32}P]- or [γ-^{32}P]- ATP (Perkin Elmer, Waltham, MA), 500 μM PPi (sodium salt). A total of 5 μM ubiquitin was added to the mixture to initiate the ATP:AMP exchange. After incubation at 30°C for 10 minutes, the reactions were quenched with EDTA, and 0.5 μl aliquots of the reaction mixtures were spotted on Baker-flex® thin layer chromatography (TLC) polyethylenimine-modified cellulose plates (J.T. Baker, Phillipsburg, NJ) and developed in filtered 0.34 M potassium phosphate pH 7.0 for 20 minutes in a glass jar. The TLC plates were allowed to air dry for 10 minutes, covered in plastic wrap, and then exposed to a phosphoimager plate for 5–10 minutes. The separation of radiolabeled nucleotides was visualized using a Typhoon scanner 9400 (GE Healthcare, Piscataway, NJ).

Results

Largazole stabilizes GFP-p27 expression in Kip16 cells

A hallmark of many advanced cancers is an excessive degradation of the cyclin-dependent kinase inhibitor p27, which is chiefly directed by SCF^{Skp2}-mediated ubiquitination. Hence, stabilization of p27 degradation represents a rational approach in cancer therapeutics. To identify small molecule inhibitors that can stabilize p27Kip1, we performed a screen of ~3000 compounds from NCI DTP diversity set along with several natural products in our collection. For the cell-based screen, we generated a mink lung epithelial cell line (Kip16) stably expressing p27 that was cloned in frame with green fluorescent protein (GFP). The resulting N-terminal GFP-p27 fusion, detectable by fluorescence microscopy, was used to determine the levels of p27 expression upon treatment of cells with the compound libraries in 96-well format. Much to our surprise, the most potent hit that emerged from this screen was the natural compound Largazole (L) (Figure 1), which was first described by Luesch and coworkers [21] and subsequently synthesized in several laboratories including ours [21,24–26,29,30,32]. Largazole induced a robust and highly uniform upregulation of GFP-p27 at concentrations as low as 1 nM (Figure 2A). As expected, treatment with the proteasome inhibitor MG132 is highly effective in prevention of p27 degradation. We did not observe an increase in GFP-p27 expression upon treatment with the vehicle DMSO. This result suggests that Largazole can stabilize GFP-p27 expression in cultured cells.

Largazole and select analogs inhibit the *in vitro* ubiquitination of p27 and Trf1

Before Largazole's function as an inhibitor of histone deacetylase was revealed, our initial investigation into the mechanism of this compound showed its ability to impede degradation of GFP-p27 in Kip 16 cells. One way to stabilize p27 is to block its ubiquitination. Hence we hypothesized that Largazole stabilizes p27 by inhibiting the ubiquitination pathway [7,8]. One of the downsides of cell-based assays is that the effects observed may be attributed to the influence of multiple pathways. For example, inhibiting the proteasome, elevating transcription of p27, or inhibiting Cdk activity can also lead to an increase in p27 expression. To tease out the mechanism and action of Largazole on p27 stabilization, we decided to test the effect of Largazole on p27 ubiquitination in a fully reconstituted system *in vitro* [33,35]. To test if Largazole affects p27 ubiquitination *in vitro*, we added Largazole to a p27 ubiquitin ligation reaction. As shown in Figure 2C, adding Largazole significantly reduced polyubiquitinated p27, suggesting that Largazole blocks p27 ubiquitination. Since Largazole is known to be a histone deacetylase inhibitor and has a thioester moiety that links an aliphatic chain to the core, we decided to test whether inhibition of p27 degradation can be linked to its histone deacetylase inhibitory activity. The structure-activity relationship for Largazole is relatively well understood [36]. Therefore we next tested a series of Largazole analogs (Figure 1) to study the effect of structure-activity relationship on p27 ubiquitination. To investigate this, Largazole ester (E), Largazole ketone (K), Largazole macrocycle (M), and *seco*-Largazole (S) were tested in an *in vitro* p27 ubiquitination assay (Figure 2C). We also added the HDAC inhibitor Trichostatin A (TSA), the structure of which can be found in Figure 1, to the assay to determine whether or not other HDAC inhibitors affect p27 ubiquitination. We observed that Largazole (L), Largazole ketone (K), and Largazole ester (E) inhibited the ligation of ubiquitin onto p27; however, the M and S analogs and TSA failed to inhibit the ubiquitination of p27 (Figure 2C). The fact that M had no inhibitory activity highlights the role of the octanoyl chain in hindering p27 polyubiquitination. *Seco*-Largazole (S) did not affect p27 ubiquitination, indicating the importance of the topology of the inhibitory compound. Furthermore, the result also suggests that the thioester moiety of Largazole is not required for inhibition, because the ketone and ester analogs were equally potent in blocking p27 ubiquitination. In addition, E1 inhibition is

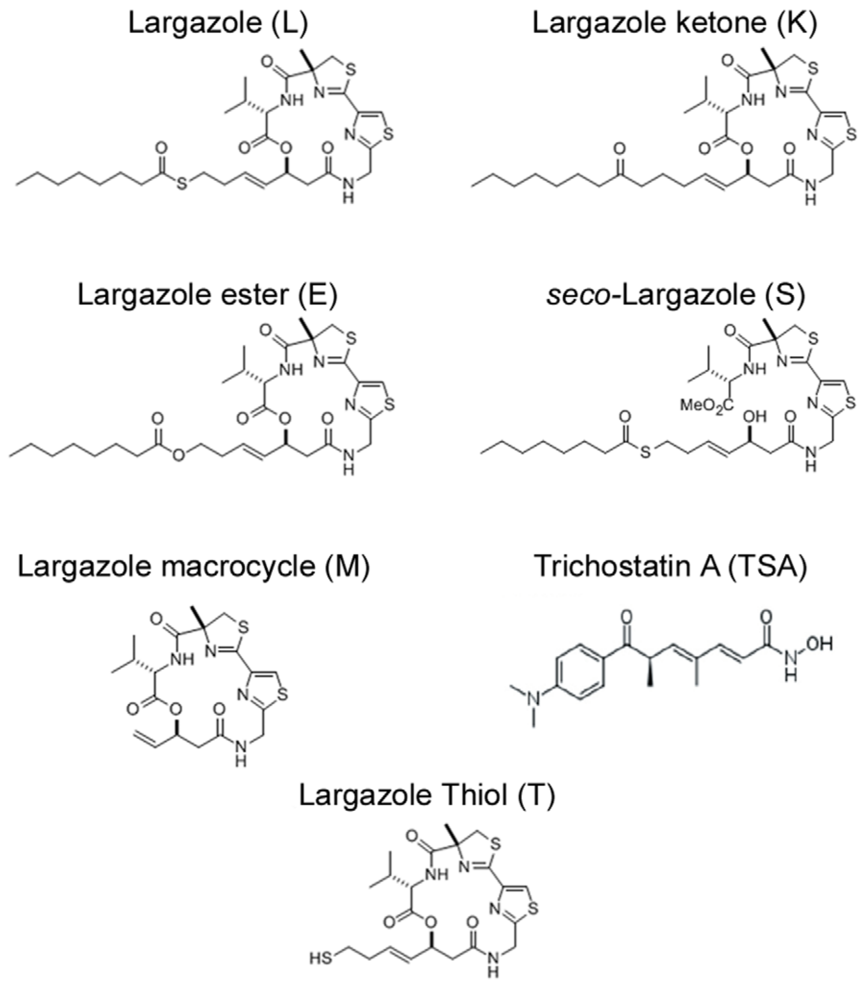

Figure 1. Chemical structures of Largazole, synthetic analogs, and Trichostatin A. Largazole (L) includes a substituted 4-methythiazoline linearly fused to a thiazole, a 3-hydroxy-7-mercaptohept-4-enoic acid, a thioester moiety, and a hydrocarbon tail. Analogs include a substituted ketone (K) and ester (E) in place of the thioester moiety, a macrocycle lacking the thioester moiety and hydrocarbon tail (M), an analog containing a macrocycle broken at carbon-3 of the enoic acid (S), and a thiol analog lacking the thioester moiety (T). Trichostatin A (TSA) contains a hydroxamic acid functional group.
doi:10.1371/journal.pone.0029208.g001

unrelated to HDAC inhibitor activity of Largazole as both ketone and ester fail to inhibit HDAC. Prior to ubiquitination, p27 is phosphorylated by the Cdk2-CyclinE complex. We carried out an *in vitro* p27 phosphorylation assay (as described in [33]) in the presence of either DMSO or Largazole in order to test whether or not the decrease in p27 ubiquitination was due to the inhibition of the Cdk2-CyclinE complex. We observed that Largazole does not inhibit the phosphorylation of p27 (Figure 2B); therefore, the inhibition of p27-ubiquitin conjugation is due to an inhibition of the ubiquitination process rather than phosphorylation step.

To study the specificity of largazole's inhibition, we set up Trf1 *in vitro* polyubiquitination in the presence of varying concentrations of largazole ester and found that E inhibited the ubiquitin attachment in a dose-dependent manner (Figure 2D). Since ubiquitination of both proteins was impeded, and given that both reactions require different factors to execute it (p27 has to be phosphorylated by CDK2-CyclinE while there is no requirement for Trf1 phosphate addition, E2 for p27 is Cdc34, while Trf1 needs Ubc5Ha, SCFSkp2 ligase works with p27 while Fbx4 is the substrate recognition subunit for Trf1) it was evocative that largazole compounds stall a step that is common to both polyubiquitination reactions.

Largazole and ester/keto analogs inhibit ubiquitin E1 activation

Since both p27 and Trf1 can be ubiquitinated in the presence of UBA1, we hypothesized that the inhibitory activity of Largazole is due to the deactivation of E1. To test this hypothesis, we incubated Largazole and Largazole ester with recombinant E1 prior to carrying out an *in vitro* thioester assay we described previously [37]. In addition, we tested the active thiol form of Largazole (T) for E1 inhibition. The presence of a fluorescence signal in the thioester assay suggests the formation of E1-ubiquitin adducts. The dose dependent decrease in fluorescence indicates that Largazole and Largazole ester inhibit the formation of E1-ubiquitin adducts (Fig. 3AC). The dose-response curves generated (data not shown) suggest an IC_{50} of approximately 29 μM and 25 μM for Largazole and Largazole ester, respectively. Interestingly, the active thiol form of Largazole (T) failed to inhibit E1 (Fig. 3G), suggesting again that the octanoyl residue is important for inhibition.

Activated ubiquitin is normally transferred to ubiquitin conjugating enzymes (E2). If E1 activity is inhibited, we expect to see that defects in E1 activation should impair the attachment of

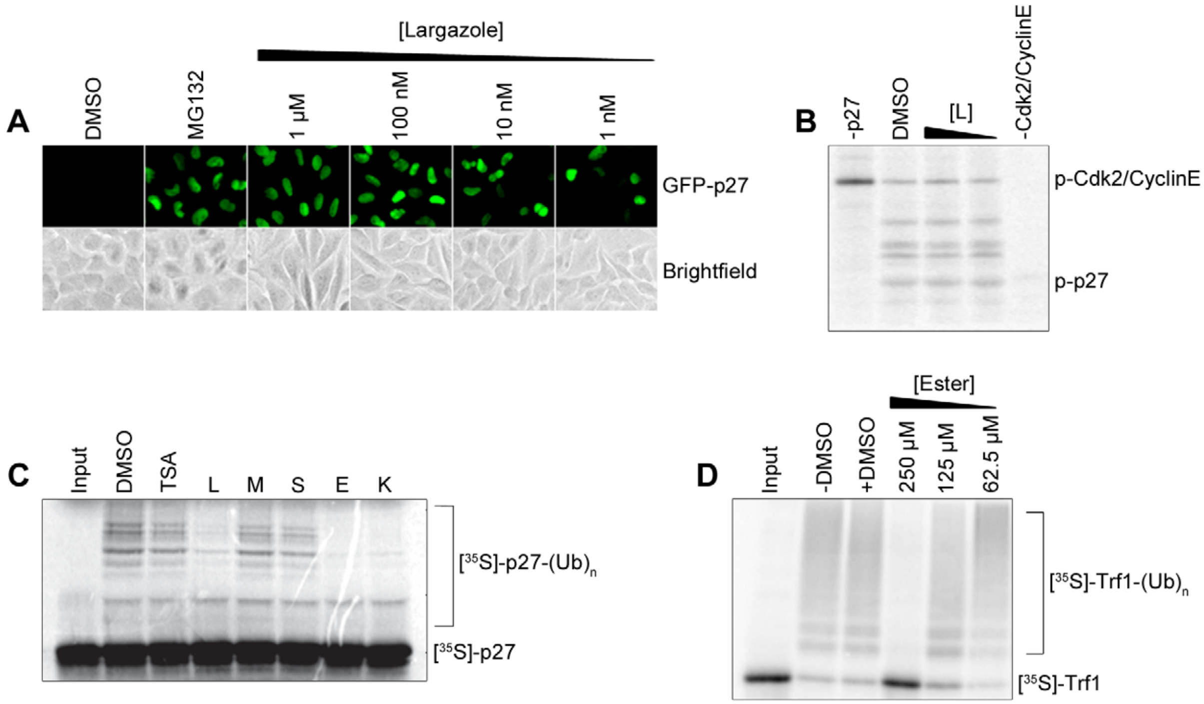

Figure 2. Largazole stabilizes p27 expression in Kip16 cells and inhibits p27 ubiquitination *in vitro.* (A) Fluorescent and corresponding bright-field images of Kip16 cells treated with varying concentrations of Largazole (L). L treatment induces the expression of GFP-p27 in a dose-dependent fashion. Addition of MG132 (1 μM) prevents the degradation of GFP-p27 via the ubiquitination and subsequent proteasomal degradation pathway. The vehicle control, DMSO, has no effect on the reporter protein stabilization. (B) L fails to inhibit the phosphorylation of p27 by the Cdk2/CyclinE complex compared to the positive control. L (250 μM, lane 3, and 125 μM, lane 4) was incubated with the Cdk2/CyclinE complex prior to the autophosphorylation of Cdk2/CyclinE step. Phosphorylated-p27 was identified by protein standard. (C) L, K, and E reduce polyubiquitinated forms of p27 while M and S have no inhibitory effects. Ubiquitin-activating enzyme E1 (100 nM), UBA1, was incubated with 100 μM of each compound prior to the reaction. (D) E reduces polyubiquitinated forms of Trf1 in a dose-dependent fashion. UBA1 (100 nM) was incubated with either DMSO or various concentrations of E ranging from 250 μM to 1 μM prior to the reaction.
doi:10.1371/journal.pone.0029208.g002

ubiquitin onto Cdc34 (E2). To further validate E1 inhibition, we included Cdc34, the E2 enzyme required for p27 ubiquitinaton, in the E1 reaction mixture. As shown in Figure 3EF, in the presence of ATP, fluorescent ubiquitin is transferred to Cdc34 indicated by the presence of a fluorescent Cdc34 band on the gel. Upon incubation with E1, Largazole or Largazole ester reduce the amount of ubiquitin molecules that are transferred from E1 to E2 in a dose-dependent fashion (Fig. 3BD).

The decreased ubiquitin transfer could be attributed to either E1 or E2 inhibition; therefore, we produced E1 precharged with ubiquitin by incubating ATP and fluorescent ubiquitin for 15 minutes at room temperature followed by the addition of Cdc34, which was preincubated with either Largazole or Largazole ester. If either compound inhibits the transfer of ubiquitin from E1 to E2, then we would observe a significant decrease in Cdc34 fluorescence regardless of the order we added the compounds. Interestingly, Largazole, preincubated with Cdc34, fails to inhibit the transfer of ubiquitin from precharged E1 at concentrations <1 mM (Fig. 3E). Furthermore, in a similar experiment, Largazole ester begins to inhibit the transfer of ubiquitin from precharged E1 to Cdc34 at concentrations around 500 μM (Fig. 3F), although this concentration is significantly above the IC_{50} of E1 inhibition. These results suggest that Largazole and Largazole ester exhibit selectivity towards ubiquitin E1. Also, this result suggests that either compound fails to promote the hydrolysis of ubiquitin thioesters on precharged E1.

Largazole ketone inhibits the adenylation step of E1 activation

E1 forms an ubiquitin–adenylate intermediate during the course of its catalytic cycle [3]. Thus the mechanism of ubiquitin E1 activation can be studied by assaying ATP:PPi and ATP:AMP exchanges [3]. Production of AMP in the [α-^{32}P]-AMP:[α-^{32}P]-ATP exchange assay guarantees that a thioester bond is formed between E1 and ubiquitin, while the release of PPi, measured by the [^{32}P]-PPi:[γ-^{32}P]-ATP exchange assay, signals the formation of ubiquitin adenylate. To further dissect the mechanism of Largazole inhibition, two nucleotide exchange assays were carried out in the presence of Largazole derivatives. For these experiments we used Largazole ketone, which is similar to Largazole and Largazole ester. From the results shown in Figure 4, it is evident that the first two concentrations of Largazole ketone (100 and 50 μM) inhibit ubiquitination of E1 similarly and were also inhibitory in both types of exchange assays. The lack of a [^{32}P]-PPi signal suggests that the adenylation step did not occur; consequently, ubiquitin could not be transferred to the active site cysteine to trigger the release of AMP. Both steps of the E1-catalyzed reactions can be measured by the AMP:ATP exchange assay. The lack of an [α-^{32}P]-AMP signal further suggests that the adenylation step is inhibited by Largazole ketone. Thus Largazole or Largazole derivatives act on the first step of ubiquitin activation pathway by blocking the formation of ubiquitin-adenylate.

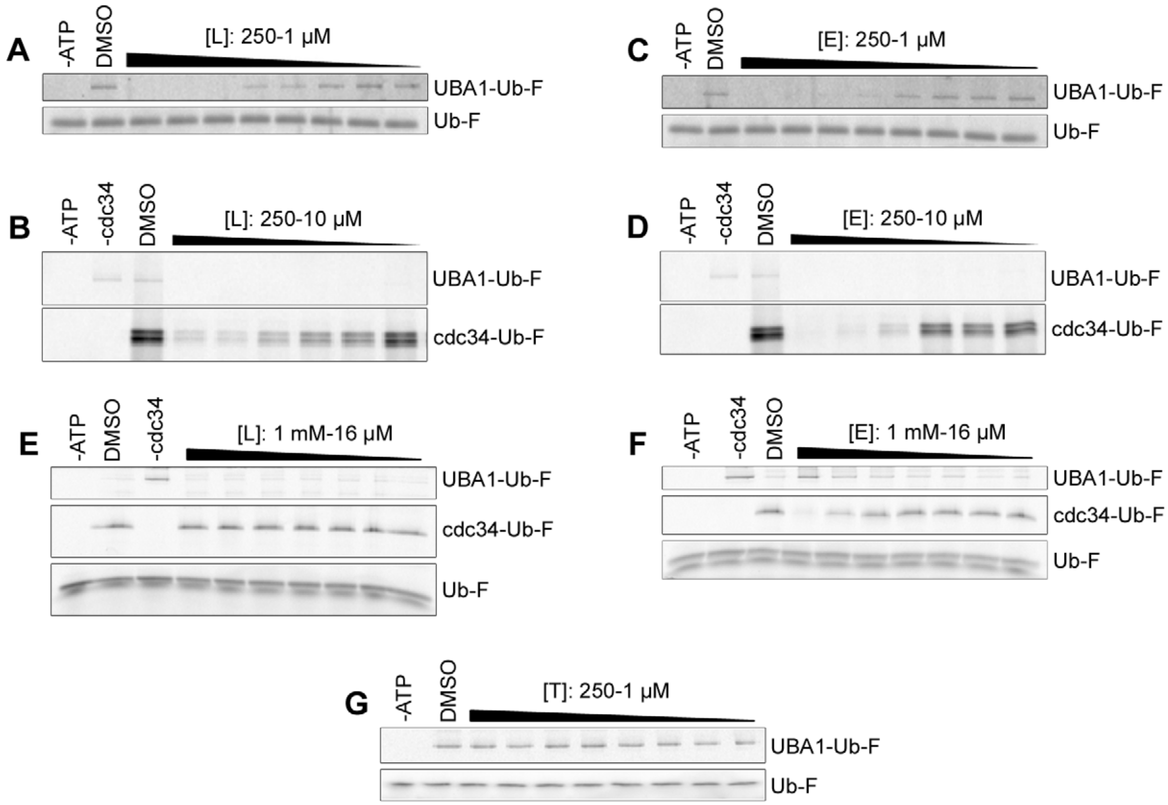

Figure 3. Largazole (L) and largazole ester (E) inhibit ubiquitin E1 in a dose dependent manner *in vitro*. (A,C) L and E inhibit transfer of ubiquitin onto E1 in a concentration-dependent manner. Thioester assay of E1 activity using fluorescein ubiquitin (Ub-F). Thioester bond formation between E1 and Ub-F is ATP-dependent (lane 2 vs. lane 1). In addition, DMSO has no effect on the formation of the thioester linkage as seen in lane 2 of both gels. 50 nM E1 was incubated with decreasing concentrations of L (A) or E (C) for 15 minutes at room temperature followed by addition of a cocktail containing ATP and Ub-F. After 5 minutes of incubation, the reactions were resolved by SDS-PAGE under non-reducing conditions. Ub-F was used to show equal loading. (B,D) Thioester assay of the ubiquitin transfer from E1 to E2 (Cdc34). Largazole or Largazole ester, when preincubated with 50 nM E1 for 15 minutes, inhibit the transfer of ubiquitin from E1 to Cdc34 in a concentration-dependent manner. (E) Largazole selectively inhibits the activity of E1 not E2. 50 nM E1 was pre-charged with ATP and then added to Cdc34 that was previously incubated with decreasing concentrations (1 mM–16 μM) of L in thioester reaction mixture. (F) Largazole ester inhibits E2 at high concentrations. Pre-charged E1 was added to reactions that contained Cdc34 pre-incubated with E ranging from 1 mM to 16 μM and resolved by SDS-PAGE under non-reducing conditions. Complete inhibition of ubiquitin transfer to E2 was observed at 1 mM of E, with only modest inhibition at 500 μM. (G) Largazole thiol (T) has no effect on transfer of ubiquitin onto E1. The reaction was carried out as described in A,C.
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Selectivity of Largazole ketone against SUMO E1 and Uba1p

In addition to ubiquitin, there exist several ubiquitin-like proteins that covalently modify other proteins. All of the ubiquitin-like proteins have activation pathways similar to ubiquitin [38]. In order to study the specificity of Largazole to the ubiquitin pathway, we incubated Largazole ketone with SUMO-activating E1 enzyme prior to carrying out a thioester assay. From the results in Figure 5B, we found that Largazole ketone is ineffective in inhibiting the formation of E1-SUMO adducts. From the dose-response curve generated from the SUMO E1 fluorescence results, the IC_{50} is approximately 450 μM as opposed to 30 μM for ubiquitin E1 (data not shown). Thus Largazole is relatively selective in perturbing ubiquitin E1 activation.

Ubiquitin and the ubiquitin E1 enzyme are highly conserved among eukaryotes [38]. Sequence analysis shows a 45% homology between the human ubiquitin-activating enzyme E1 (UBA1) and *S. pombe* E1 (ptr3/Uba1p) at the amino acid sequence level. To test whether Largazole ketone inhibits the *S. pombe* E1, we carried out a thioester assay using Largazole ketone and the ubiquitin E1 homologue in *S. pombe*, Uba1p. The results in Figure 5A suggest that Largazole ketone fails to inhibit the formation of E1-ubiquitin adducts at concentrations less than 1 mM. Taken together, these results suggest that Largazole and its derivative are highly selective in inhibiting the ubiquitin E1 enzyme.

Discussion

In this study, we showed that Largazole and its analogs selectively inhibit ubiquitin E1 enzyme activity *in vitro*. Also, we demonstrated that the inhibitory activity of Largazole is independent of its inhibitory activity towards the histone deacetylase enzymes. Structure-activity relationship analysis shows that the thioester bond is not required for inhibition but the macrocycle core and aliphatic tail are indispensible. Largazole blocks ubiquitin activation at the adenylation step and without perturbing ubiquitin transfer from E1 to E2. Finally we show that Largazole inhibition of E1 is highly selective as it does not inhibit a highly related ubiquitin E1 enzyme from *S. pombe* and is almost twenty fold less effective in inhibiting the activation of SUMO E1. Taken together, our results reveal that Largazole represents a new class of ubiquitin E1 inhibitors.

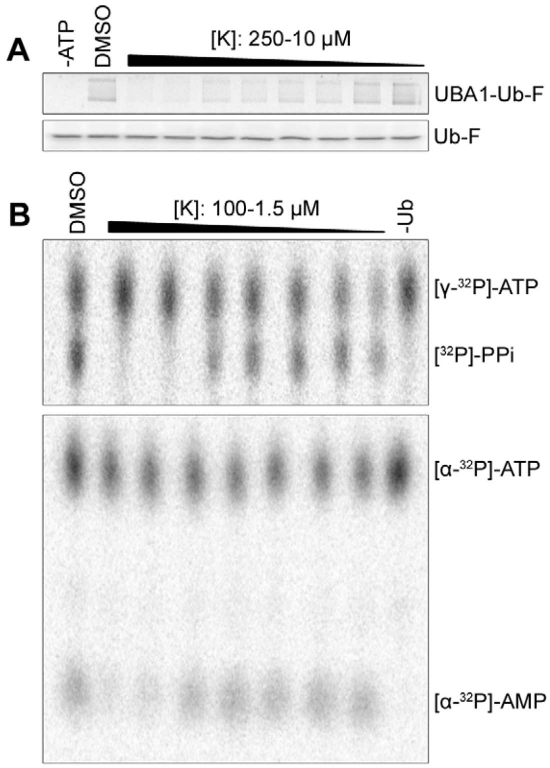

Figure 4. Largazole ketone inhibits the adenylation of the E1 ubiquitin-activating enzyme. (A) Largazole ketone inhibits ligation of ubiquitin onto E1 in a concentration-dependent fashion. Reduction of E1~Ub adducts was determined by thioester assay utilizing fluorescein ubiquitin. (B) Largazole ketone inhibits the adenylation step in ubiquitin E1 activation in a concentration-dependent fashion. K was serially diluted (100 μM to 1.5 μM) and incubated with UBA1 (150 nM) at room temperature for five minutes. The thioester reaction mixture was mixed with ubiquitin to initiate the PPi:ATP exchange (middle panel) or AMP:ATP exchange (bottom panel) and added to the UBA1/K mixture. All reactions were halted with addition of EDTA after 10 minute incubation at 37°C, resolved using Cellulose PEI TLC plates, and analyzed using a phosphoimager.
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We identified that Largazole caused a robust increase in GFP-p27 expression in Kip16 cells. This observation led us to further investigate the mechanism of GFP-p27 stabilization by Largazole. Using an *in vitro* ubiquitination assay, we were able to delineate the inhibitory point where Largazole acts on in the ubiquitination pathway, namely the E1 enzyme. However, there is a disconnect between the potency of E1 inhibition *in vitro* and GFP-p27 stabilization in cells. The EC_{50} of Largazole for GFP-p27 stabilization is in the low nM range, yet E1 inhibition is at ~30 μM. This results suggests that the stabilization of GFP-p27 is unlikely caused by E1 inhibition, but is most likely a result of HDAC activity, which is known to block cell cycle progression and cause cell growth arrest. Consistent with this hypothesis, Largazole ketone and ester, two Largazole analogs that do not inhibit HDACs, do not increase GFP-p27 levels when Kip16 cells were treated (data not shown). However, other interpretations may account for the failure of Largazole ketone or ester to raise GFP-p27 by inhibiting E1 in cells. For example, we do not know if or how these compounds penetrate cells and how stable they are once they enter the cells. These investigations have to be undertaken before these analogs can be further developed for *in vivo* applications.

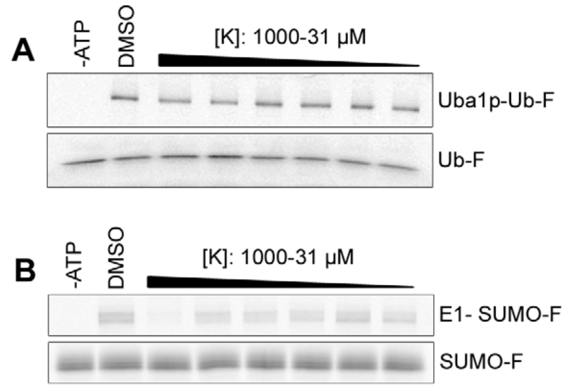

Figure 5. Investigation into the selectivity of Largazole ketone. (A) Largazole ketone (K) fails to inhibit the ligation of ubiquitin onto Uba1p, a homologue of UBA1 from *S. pombe*. Formation of Uba1p-ubiquitin adducts was determined by thioester assay utilizing fluorescein-ubiquitin. Uba1p (1.03 μM) was incubated with either DMSO or various concentrations of K serially diluted from 1000 μM to 31 μM. (B) K inhibits ligation of SUMO-1 onto human SUMO E1 in a concentration-dependent fashion. Reduction of E1-SUMO adducts was determined by thioester assay utilizing fluorescein-SUMO-1. hSUMO E1 (500 nM) was incubated with either DMSO or various concentrations of K serially diluted from 1000 μM to 31 μM.
doi:10.1371/journal.pone.0029208.g005

Panepophenanthrin, a natural compound derived from the mushroom strain *Panus rudis*, and Himeic acid A, derived from the marine fungus *Aspergillus*, are the first and second discovered inhibitors of the ubiquitin-activating enzyme E1, respectively [39,40]. Both compounds were tested *in vitro* using recombinant E1; however, the cellular activity and mechanism were not determined [41]. PYR-41 and related pyrazones are another set of compounds that were discovered to inhibit ubiquitin E1 and the first set of E1 inhibitors described to enter cells and differentially kill transformed cells [42]. The IC_{50} of PYR-41 is around 5 μM, thus more potent than the compounds described here. However, the exact mechanism of PYR-41 inhibition is not known. Ub-AMSN represents a distinct class of protein based inhibitors of ubiquitin E1. Ub-AMSN contains a sulfamide group attached to the carboxyl terminus of ubiquitin as a nonhydrolyzable mimic of the phosphate group in the cognate Ub/Ubl-AMP adenylate intermediate. Thus, like Largazole analogs, it blocks the first step of E1 reaction [43,44]. Unfortunately, Ub-AMSN cannot be used in cells as it cannot pass through the cell membrane. However, Ub-AMSN turns out be a very useful for probing the structure and biochemical mechanisms of E1 enzyme [44]. Therefore, Largazole and analogs could also be useful tools for probing ubiquitin function.

One of the most important questions to be answered is whether or not ubiquitin or ubiquitin-like E1 inhibitors are therapeutically relevant. Since only one ubiquitin E1 enzyme is responsible for a majority of protein ubiquitination in humans, inhibiting E1 will influence the degradation of proteins across several pathways and may lead to toxicity and, consequently, poor therapeutic efficacy. Bortezomib is the first FDA-approved proteasome inhibitor for the treatment of relapsed/refractory myeloma and mantle cell lymphoma. [19]. The proteasome, particularly the 26S proteasome, is the final step in ubiquitin-mediated protein degradation and regulates various pathways necessary for cellular function. The clinical success and efficacy of Bortezomib gives rise to the possibility that inhibitors of ubiquitin E1 will also share similar success. NEDD8 is a protein modifier that shares mechanistic and structural similarities to ubiquitin. Currently, the cullin family of

proteins has been characterized as the target for NEDD8 conjugation [11]. MLN4924 is a potent and selective inhibitor of the NEDD8-activating enzyme (NAE) that exhibited potent cytotoxicity against several human tumor-derived cell lines [45]. Interestingly, MLN4924 shares a similar mechanism to Largazole analogs. MLN4924 reacts covalently with NEDD8 mimicking a NEDD8 adenylate that is incapable of driving the reaction forward, therefore, blocking the activity of NAE [46]. MLN4924 is currently undergoing phase I clinical trials in patients with lymphoma, multiple myeloma, or any form of nonhematologic malignancies. The *in vitro* and possible clinical success of the NAE inhibitor MLN4924 further supports the concept that E1 inhibitors are potential promising cancer therapeutics.

Our preliminary structure activity relationship studies suggest that the pro-drug form of Largazole including both the hydrocarbon tail and the macrocycle are essential for E1 inhibition. For Largazole analogs to be developed as potential antitumor drugs, additional analogs are needed to be synthesized in order to improve its potency toward ubiquitin E1. The most promising aspect of Largazole analogs as ubiquitin E1 inhibitors is the selectivity and specificity of Largazole. Largazole analogs not only display discrimination over related SUMO E1 enzyme but also remarkable selectivity in targeting human ubiquitin E1. Future structural studies would be helpful to understand how Largazole analogs inhibit E1, and insights gained from such studies may help to develop more specific inhibitors of E1. Experiments to test these hypotheses are currently underway.

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Author Contributions

Conceived and designed the experiments: DU SJP. Performed the experiments: DU SJP WW. Analyzed the data: DU SJP. Contributed reagents/materials/analysis tools: CGN BQ GZ RDK AJP. Wrote the paper: SJP DU XL.

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